

Distribution, Ecology and Cytology of *Asplenium azoricum* Lovis, Rasbach & Reichstein (Aspleniaceae, Pteridophyta) and Its Hybrids

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ABSTRACT.—New data on distribution, ecology and cytology of the Azorean endemic *Asplenium azoricum* are presented. Hybrids involving this species are recorded for the first time. The extremely rare, natural hybrid *A. azoricum* × *A. scolopendrium* is described as *A. ×santamariae*. It has been found at a single location on Santa Maria Island and is presumed to be triploid. A more frequent natural hybrid *A. azoricum* × *A. onopteris*, found at seven locations on the same island, is also new to science and is described here as *A. ×diasii*. It is confirmed cytologically as triploid with two genomes from *A. azoricum* and one from *A. onopteris*. *Asplenium ×diasii* and an experimental hybrid show that *A. azoricum* is an allotetraploid species. The parentage and directionality of hybridization for both hybrid taxa have been established using uniparentally inherited plastid genome markers.

The Azores is an isolated archipelago of nine inhabited, volcanic islands in the Northern Atlantic Ocean. The shortest distance to the European coast, Cabo da Roca, Portugal, is almost 1,300 km. The American coast, Newfoundland, is about 1,700 km distant (Fig. 1).

Four morphologically similar, simply-pinnate species of the genus *Asplenium* have been reported in the Azores: the almost cosmopolitan tetraploid *A. trichomanes* L. ssp. *quadrivalens* (D. E. Mey.) Lovis; the widely scattered Neotropical triploid apomict *A. monanthes* L.; the locally rare Macaronesian endemic diploid *A. anceps* Lowe ex Hook. & Grev. (Rasbach *et al.*, 1981), two plants of which were reported from Pico island in 1973 (Lovis *et al.*, 1977) and the endemic tetraploid *A. azoricum* Lovis, Rasbach & Reichstein.

Although *A. azoricum* was collected by K. Hochstetter in 1838 on Faial Island (Seubert, 1844), its specific distinction was not recognized until the work of Lovis *et al.* (1977). Key distinguishing features had already been commented upon by Wilmanns and Rasbach (1973), and earlier authors, e.g., Milde (1867), and Trelease (1897), obviously recognized that Azorean material differed from typical *A. anceps*, referring their gatherings to a forma (f. *azorica* Milde) of that species. While previously confused with both *A. anceps* and *A. trichomanes*, its separation in the field generally is quite easy. *Asplenium azoricum* differs from both in the more elongated, broadly-triangular, often

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biauriculate, more conspicuously dentate pinnae, and from the latter in its glossier, bright green frond colour. Typical luxuriant material from shaded, sheltered environments is considerably larger than any of the taxa listed above, with fronds sometimes exceeding 35 cm long. Confusion is possible, particularly with *A. trichomanes* ssp. *quadrivalens*, when comparing small examples from dry, exposed environments. *Asplenium anceps*, which is implicated in the parentage of *A. azoricum* but currently is not sympatric with it, differs in having a very pronounced additional third rachis wing on the abaxial side. A greater potential confusion is with another recently described taxon, *A. trichomanes* ssp. *coriaceifolium* H. & K. Rasbach, Reichstein & Bennert (syn.: *A. azomanes* Rossello, Cubas & Rebassa). This highly restricted tetraploid was described from walls terracing olive groves in the Balearic Islands, where it is also found in sheltered rocky gulleys of seasonal watercourses close to sea-level. It, however, also occurs on montane karstic limestones in Southern Spain and the Rif mountains of Morocco (Rumsey and Vogel, unpubl.). Its genomic constitution and relationship to *A. azoricum* are currently being explored.

Natural hybrids of *A. azoricum* have not previously been reported (Reichstein, 1981) although they have been actively searched for (Lovis *et al.*, 1977). Plastids have been shown to be maternally inherited in *Asplenium* (Vogel *et al.*, 1998a), and fragment length polymorphism and sequence data for this moderately variable plastidic region allows for unequivocal identification to the species level (Vogel *et al.*, unpubl.). Thus, molecular studies of this sort facilitate both the determination of hybrid parentage and the establishment of hybrid directionality, i.e., which parental taxon was maternal.

MATERIAL AND METHODS

The distribution and ecology of *A. azoricum* was studied between 1998 and 2001 on the nine islands of the archipelago, during field work by H.S. for a project mapping all vascular plant species. Distribution maps based on the UTM 1 × 1 km grid have been created for islands representative of the western, central and eastern groups of the archipelago: Flores, Faial and Santa Maria (Fig. 2).

During the course of our fieldwork, a number of plants with abortive spores were detected whose morphology suggested possible hybridity. Fronds with premature sporangia were fixed in a mixture of acetic acid and ethanol (1:3) in the field. Preparations of meiosis were made following Manton (1950). A few mature fronds of each potential hybrid were collected as voucher specimens and will be deposited in the herbaria of Universidade dos Açores, Terceira (AZU) and Natural History Museum, London (BM). Live material was not collected as all hybrids are rare and should be protected in their natural habitat on the islands.

Total DNA extractions were made from small (c. 20 mg) portions of each of the herbarium specimens using the method of Rogers & Bendich (1994) and amplified using the universal plastid primers C and F of Taberlet *et al.* (1991).



FIG. 1. Location of the Azores in the Atlantic Ocean.

RESULTS

ASPLENIUM AZORICUM LOVIS, RASBACH & REICHSTEIN.—*Asplenium azoricum* (Fig. 5a) was found on all nine islands of the archipelago, the only member of the *A. trichomanes* group for which this is true. Unlike *A. trichomanes* ssp. *quadrivalens*, which in the Azores is largely restricted to human-made structures and most abundant on the most populous islands, *A. azoricum* is widely distributed in a range of natural and semi-natural environments. Although it has been able, like many rock ferns, to exploit built structures it is not restricted to them. Usually a species of somewhat humid environments, paradoxically it is most common and shows its widest ecological amplitude on the driest, easternmost island of Santa Maria. Here it effectively replaces *A. trichomanes* ssp. *quadrivalens* (a species with very few individual plants on the island), existing in large numbers from coastal areas up to c 575 m a.s.l. on Pico Alto (Fig. 2).

Throughout the archipelago, the species achieves its greatest abundance at lower altitudes, declining markedly above the lowland forest zone. Elsewhere in the eastern group, it is locally frequent and often luxuriant in the central low-lying valley between the major volcanic peaks of São Miguel Island. In the central group of the Azores it is more restricted to west and north-west exposed

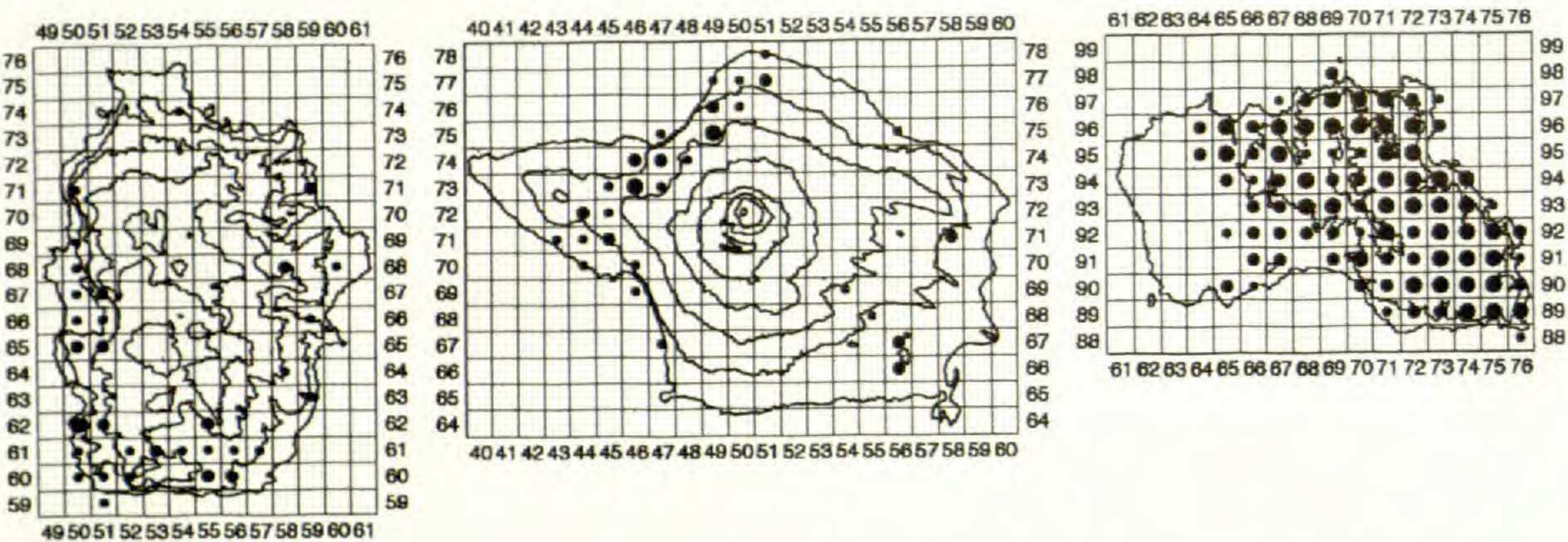


FIG. 2. Distribution of *A. azoricum* on the islands of Flores (left), Faial (center), and Santa Maria (right) in UTM 1 × 1 km grid, WGS 84 (contour lines along 200 m isohyps). Symbol size indicates abundance within that 1 × 1 km square: rare •, occasional • or common •.

slopes between 100–500 m a.s.l., although it ascends to at least 700 m on Pico (*Rumsey et al. 96-10-3-3, BM!*). In the western group, Flores and Corvo Islands, *A. azoricum* exists in the absence of *A. trichomanes* ssp. *quadrivalens*. On Flores, it is widespread (see Fig. 2) but uncommon; on Corvo, it should be considered very rare. On these islands, the species is usually found in small populations (<20 individuals), or as single plants and only at altitudes below 500 m. Restriction to particular site aspects is not so marked as in the central group, presumably because the more generally humid climate reduces the need for shelter.

Asplenium azoricum is most common on steep, humid slopes of shady ravines at low altitudes. In these places it forms large populations on bare soil, often mixed with other ferns like *A. scolopendrium* or *A. onopteris*. However, judging by earlier literature reports (e.g. *Lovis et al., 1977*) and herbarium gatherings, it is most likely to be found on or at the base of walls by roadsides and field-margins. It is one of several shade tolerant fern species that predominantly occurs in lowland areas dramatically influenced by the almost complete destruction of the natural vegetation following human arrival on the islands in the 15th and 16th centuries. *Asplenium azoricum* has fared better than some, and is now one of the very few native species that can be found in lowland forests and plantations dominated by the Australian neophyte *Pittosporum undulatum*. In this environment, it occurs in deep shade in small soil pockets on volcanic rocks on the forest floor, or rarely on the ground. In the coastal zone, it is absent in sea-wind exposed sites but grows in stands of *Erica azorica* or *Arundo donax* together with *Asplenium marinum*. Inland, while often found on mossy rocks at the base of walls, it can also be found higher on the wall proper, although typically only in soil filled crevices. A requirement for humidity throughout the year means it usually is absent from dry, south-exposed or mortared walls where it is replaced by the more xerophytic and calcicolous *A. trichomanes* ssp. *quadrivalens*. Soil backed retaining walls, or old walls which have accumulated substrate in the

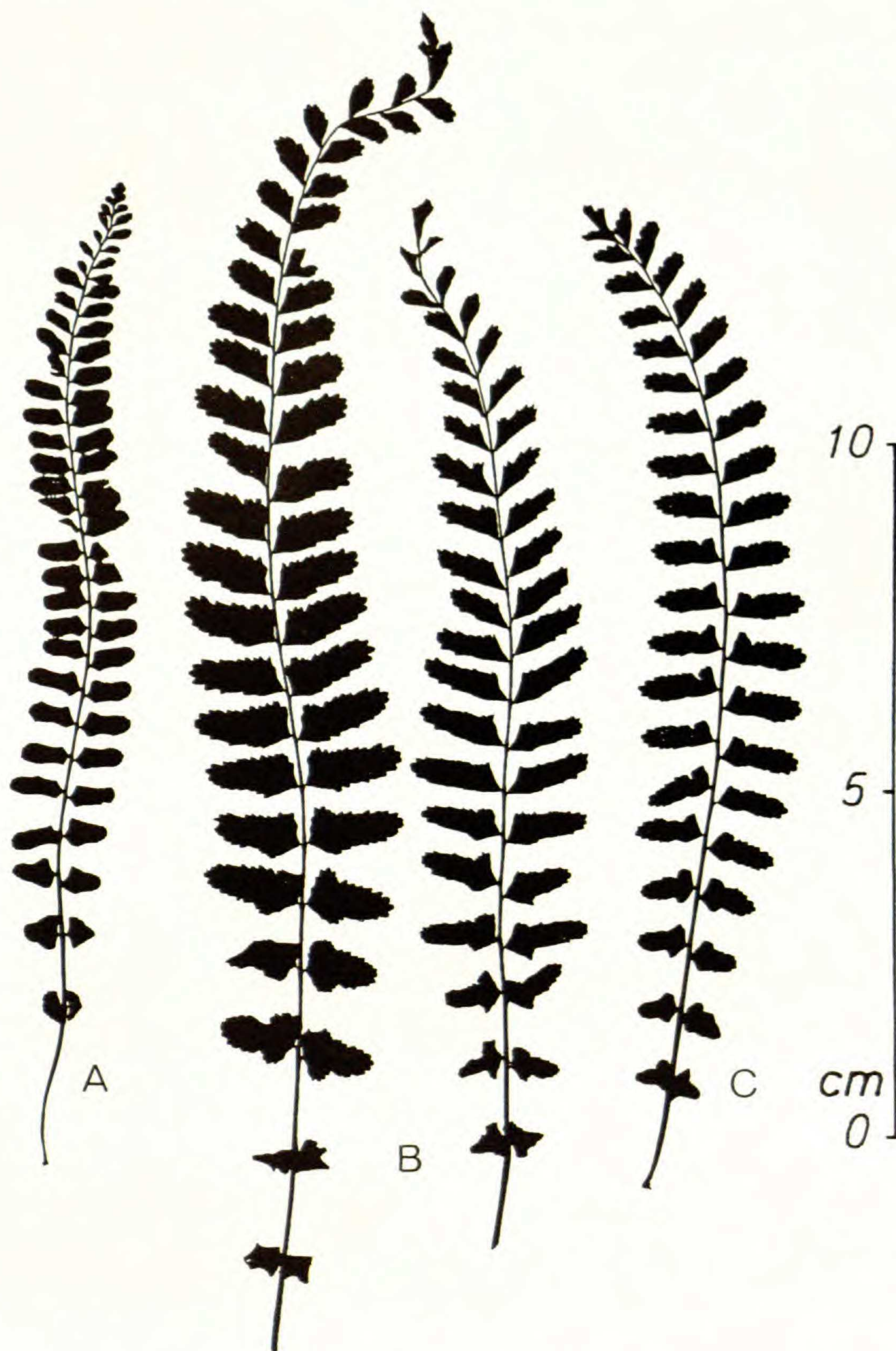


FIG. 3. Silhouettes of fronds. A. *Asplenium anceps* (Madeira, TR-2559). B. Experimental hybrid *A. anceps* \times *A. azoricum* (TR-5188 and 5188/7, in B 200125235/37, col. H. & K. Rasbach). C. *A. azoricum* (Azores, TR-3335/2). A and C from Lovis *et al.*, 1977; B- photo H.R.; TR stands for T. Reichstein.

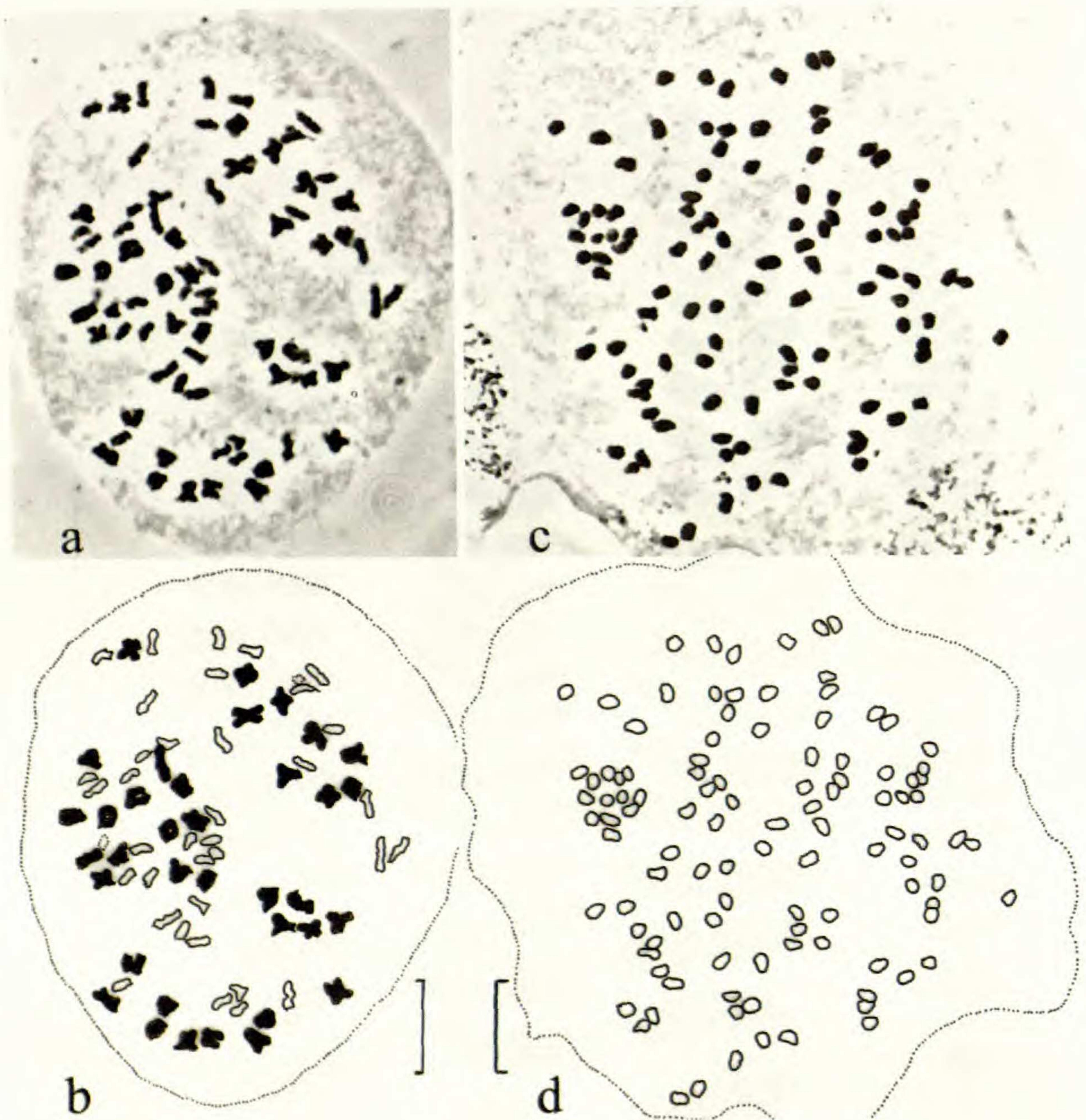


FIG. 4. A. Cytology of *Asplenium*. a. Photomicrograph of *A. anceps* \times *A. azoricum*; spore mother cell in meiosis with $n = 36\text{II}$ and 36I . b. Explanatory diagram of a; scale bar $10\ \mu\text{m}$, pairs black, univalents outlined (TR-5188, in B, collection H. & K. Rasbach: 200125235/37). c. Photomicrograph of *A. x diasii*; spore mother cell in meiosis with $n = 108\text{I}$; d. Explanatory diagram of c; scale bar $10\ \mu\text{m}$; prep. and photographs (HR Az-Ma-927 Schäfer).

interstices and are partially shaded by other vegetation, are thus most likely to support the endemic taxon. In summary, the ecological range of *A. azoricum* seems to be rather broad and with the few existing relevés it is not possible to describe vegetation associations.

Cytological investigations by Lovis *et al.* (1977) showed that *A. azoricum* is tetraploid with $2n = 144$. To determine the nature of its polyploidy and relationship to other Macaronesian taxa, a program of experimental hybridization

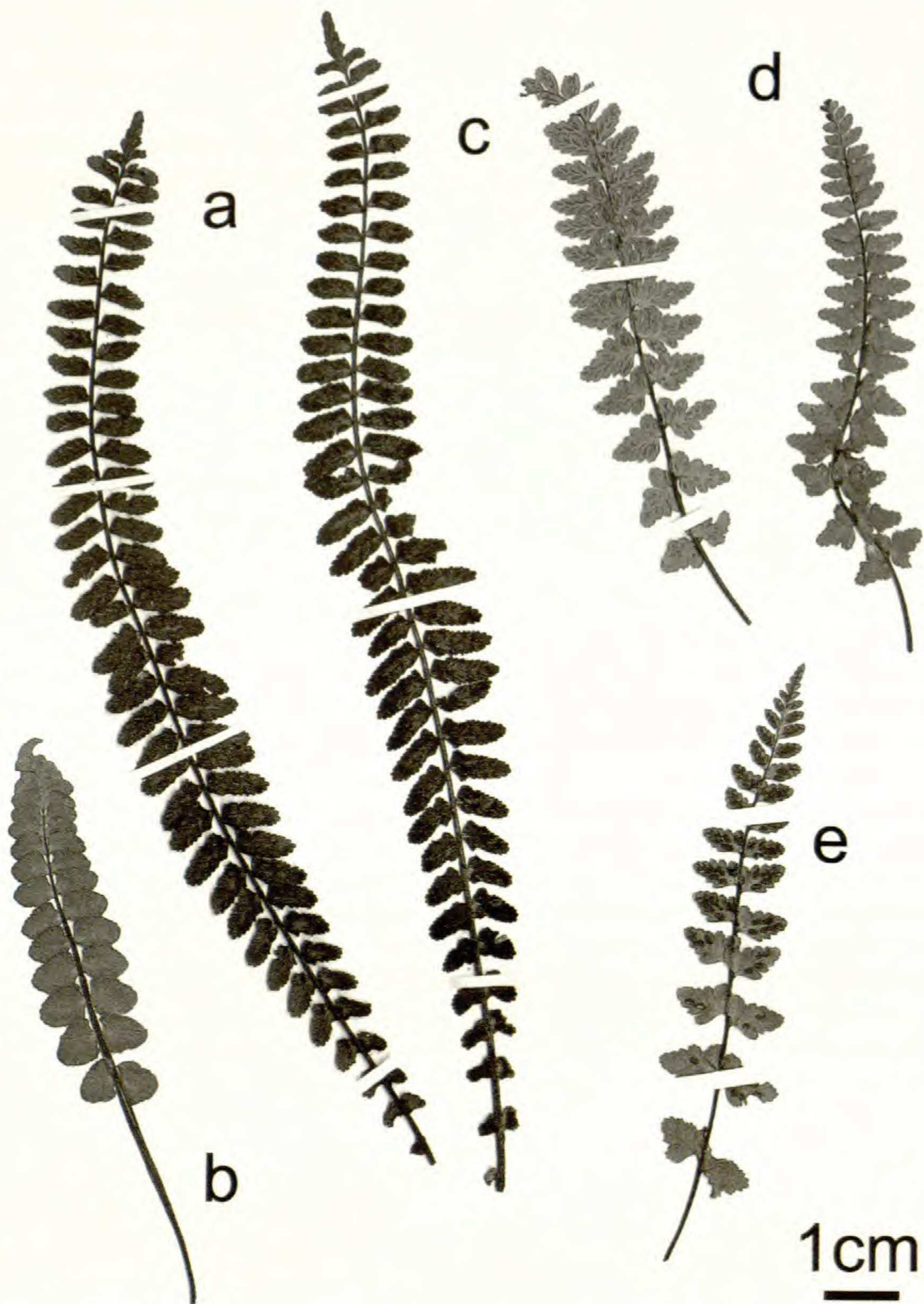


FIG. 5. Silhouettes of the fronds of *A. azoricum*, *Az-Gr-12* Schäfer, Graciosa, 2001 (a), *A. ×santamariae*, *Az-Ma-1064* Schäfer, Santa Maria, 2001 (b), and *A. ×diasii*, *Az-Ma-749* Schäfer, Santa Maria, 2001 (c), *Az-Ma-915* Schäfer, Santa Maria, 2001 (d), *Az-Ma-1036* Schäfer, Santa Maria, 2001 (e).

and cytological investigation was begun. In 1978, the late Prof. T. Reichstein hybridized *A. anceps* Lowe ex Hooker & Grev. from Madeira and *A. azoricum* from the Azores (for method see Rasbach *et al.*, 1994). This hybridization program produced 35 plants of *A. anceps*, five plants of *A. azoricum*, and 22 hybrids (fig. 3). Cytological study of one of these hybrids by Prof. J. J. Schneller (Zürich) revealed that it was triploid with $n = \text{ca. } 36 \text{ II and } 36 \text{ I}$ (Schneller, in litt. 23.01.1995). As part of this study H. Rasbach analyzed meiosis in another hybrid plant. The chromosome number of ten spore mother cells was $n = \text{ca. } 36 \text{ II and } 36 \text{ I}$ (fig. 4A).

The most parsimonious explanation of this pairing behavior is that tetraploid *A. azoricum* shares a genome with *A. anceps* and that these homologous n chromosome sets undergo synapsis in the hybrid to form the 36 pairs. The 36 unpaired chromosomes would thus represent a non-homologous genome contributed by the second diploid parent of *A. azoricum*. Under this hypothesis, *Asplenium azoricum* is an allotetraploid and chromosome pairing observed in the hybrid *A. anceps* \times *A. azoricum* is allosyndetic. The alternative hypothesis, that *A. azoricum* is an autopolyploid and that pairing in the hybrid is autosyndetic (occurring between the two genomes contributed by *A. azoricum*) can be rejected based on cytological study of the natural hybrid *A. \times diasii* reported below. We therefore conclude that *A. anceps* is one of the parental species of *A. azoricum* and suggest that the genomic constitution of this allotetraploid be represented by the formula $AnAnUnUn$, where $An = \textit{anceps}$ and Un is unknown.

On morphological grounds we hypothesize that the unknown parent of *A. azoricum* is a member of the *A. trichomanes* group *sensu lato*. Allozyme and DNA studies (Vogel *et al.*, unpublished) preclude the possibility that the missing parent ($UnUn$) is an extant European taxon. Lovis *et al.* (1977) commented on the morphological similarities with the Neotropical *A. heterochroum* Kunze. However, the material of this or similar species they examined was tetraploid and hexaploid (unpublished records), not diploid as would be required of the missing parent. Although the missing parent remains unknown, the likelihood of a Neotropical origin is perhaps strengthened by the growing list of Macaronesian cryptogamic species disjunct to the Neotropics, or with their closest relatives there. These include the pteridophytes *Ceradenia jungermannioides* (Klotzsch) R. C. Ching, *Grammitis marginella* (Sw.) Sw. (Schäfer, 2001), *Isoëtes azorica* Dur. ex Milde (Britton and Brunton, 1996), and *Huperzia dentata* (Herter) J. Holub, and the bryophytes *Jamesoniella rubricaulis*, *Radula nudicaulis* (Sjögren, 2000), *Plagiochila retrorsa* Gottsche, *P. virginica* A. Evans, *P. stricta* Lindenb., *P. papillifolia* Steph. and *P. longispina* Lindenb. & Gottsche (Rycroft, 2002).

Asplenium \times diasii Schaefer, Rumsey & Rasbach, *hybr. nov.* (*Asplenium azoricum* Lovis, Rasbach & Reichstein \times *A. onopteris* L.) TYPE:—Açores (Portugal), Ilha de Santa Maria, São Lourenço, 150 m a.s.l., in *Pittosporum undulatum* forest, $n = 108\text{I}$, triploid. 01.08.2001, H. Schäfer, Az-Ma-749 (holotype, BM). **Fig. 4c–d, 5c–e.**

Planta hybrida *Asplenio azorico* simillima; sed rhizoma caespitosa; petiolus rachisque basi ferrugineo-fuscis apice viridibus; folia 6–18 cm longa, 3–3.5 cm lata, lanceolato-acuminata, basi bipinnata; pinnae oblongo-ovatae, brevissime petiolatae, margine dentatae; sporae omnes abortivae; chromosomatum numerus 108, meiosi chromosomatibus 108 univalentibus; differt.

Rhizome caespitose. Fronds 6–18 cm, stipe to 5 cm, stipe and rachis black or dark reddish brown, the uppermost 2 cm green. Pinnae numerous (ca. 15–37), up to 16 × 12 mm, light green, short petiolate, entire or the lower bipartite, triangular to oblong-ovate, margin sharply dentate. Sori ca 1.0–3.3 mm. Triploid hybrid of the tetraploid *A. azoricum* and the diploid *A. onopteris* (maternal parent). The hybrid contains one genome of *A. onopteris* and two of *A. azoricum* that do not form pairs, i.e. are not homologous.

Named after the Azorean botanist Prof. Dr. Eduardo Dias.

PARATYPES.—Açores (Portugal), Ilha de Santa Maria: Feteiras de Cima, 220 m a.s.l., roadside slope in pasture, 13.06.2001, *H. Schäfer* Az-Ma-915 (AZU, BM) Fig 5d; Loural, 350 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer* Az-Ma-926 (AZU, BM); Loural, 350 m a.s.l., W exposed slope in pasture, n = 108I, 15.06.2001, *H. Schäfer* Az-Ma-927 (AZU, BM), (Fig. 4c); Açores (Portugal), Ilha de Santa Maria, Loural, 350 m a.s.l., *Erica* shrub on roadside, n = 108I, 15.06.2001, *H. Schäfer* Az-Ma-928 (AZU, BM); Loural, 360 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer* Az-Ma-929 (AZU, BM); Loural, 360 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer* Az-Ma-1200 (AZU, BM); NE Cruz dos Picos, roadside slope in pasture, 22.06.2001, *H. Schäfer* Az-Ma-983 (AZU, BM); Santa Bárbara, 200 m a.s.l., *Pittosporum undulatum* forest on roadside slope, 17.07.2001, *H. Schäfer* Az-Ma-1036 (AZU, BM) Fig. 5e; Feteirinha, 310 m a.s.l., E exposed slope in pasture, 24.07.2001, *H. Schäfer* Az-Ma-1072 (AZU, BM); Cardal, 350 m a.s.l., SW exposed slope in pasture, 05.08.2001, *H. Schäfer* Az-Ma-1108 (AZU, BM).

As the only simply pinnate member of the *A. trichomanes* group present in the vicinity of these hybrids, *A. azoricum* is almost certainly one of the parents. The other parent was less easily determined. Because *Asplenium* hybrids generally show clear morphological intermediacy (Wagner, 1954), the second parent was presumed to have more dissected, bi- to tripinnate fronds. In the field it was assumed that at least some of the hybrid plants could have *A. obovatum* Viv. ssp. *lanceolatum* (Fiori) P. Silva as the second parent, it being present in the majority of hybrid sites. Subsequent cytological examination revealed that the three plants investigated were triploid, not tetraploid, as would be expected from a hybrid between tetraploid *A. azoricum* and tetraploid *A. obovatum* ssp. *lanceolatum*.

Further investigation was therefore needed to establish the identity of the second parent(s). DNA extraction and PCR amplification of a portion of the plastid genome was carried out on five of the individuals identified as hybrids: *HS Az-Ma* 749, 927, 928, 1014 and 1036. Each generated identical fragments of c 1000 bp which differed markedly in their base sequence from both *A. obovatum* and *A. azoricum* but which matched that of *A. onopteris*, another

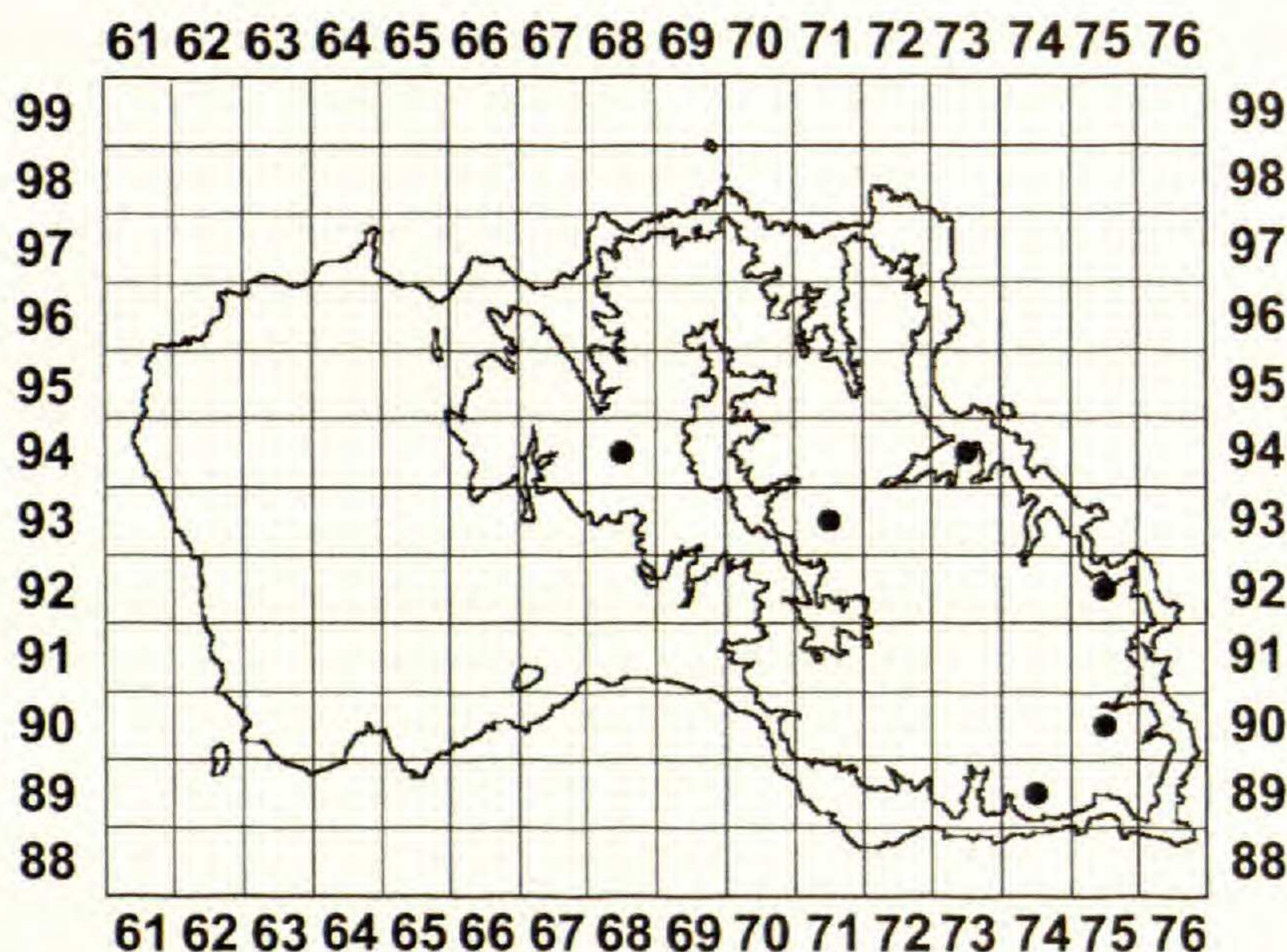


FIG. 6. Distribution of *Asplenium* \times *diasii* on Santa Maria island in UTM 1 \times 1 km grid, WGS 84 (contour lines along 200 m isohypsies).

species present at the majority of hybrid sites. The second (and consistently maternal) parent of *A. x diasii* is therefore concluded to be *A. onopteris*. This fits with the cytological data because *A. onopteris* is diploid. The considerable variability of *A. onopteris* may help explain the range of morphologies shown by the hybrid, *A. azoricum* being rather invariable. Strong unidirectionality in hybrid formation has previously been reported for *A. x alternifolium* (Vogel *et al.*, 1998b) and is true for a range of hybrids in which *A. onopteris* and its polyploid derivatives take part (Rumsey and Russell, unpubl.).

Altogether, 13 individual plants of this hybrid were found in seven locations of the eastern part of Santa Maria Island (Fig. 6). It has yet to be detected on any other island of the archipelago. The likelihood of the formation of this hybrid on the other islands is undoubtedly lessened by the reduced abundance of *A. azoricum* elsewhere. We might speculate that as the paternal species, hybrid formation is dependent on the presence of extensive growths of *A. azoricum* gametophytes and limited numbers of those of the maternal parent such that the ratio of *A. azoricum* to other species' antherozoids favors interspecific matings.

The hybrids were usually found in large mixed populations of the parents. Hybrid plants were restricted to more humid situations than the parents, often growing below them and near the foot of slopes, in communities dominated by *Pittosporum undulatum*, or *Erica azorica*.

Asplenium* \times *santamariae Schaefer, Rumsey & Rasbach, *hybr.nov.* (*Asplenium azoricum* Lovis, Rasbach & Reichstein \times *A. scolopendrium* L.). TYPE:—Açores (Portugal), Ilha de Santa Maria, Santo Espírito, 280 m a.s.l., W. exposed slope in pasture. 21.07.2001, H. Schäfer, Az-Ma-1064, (holotype, BM; isotype, AZU). **Fig. 5b**

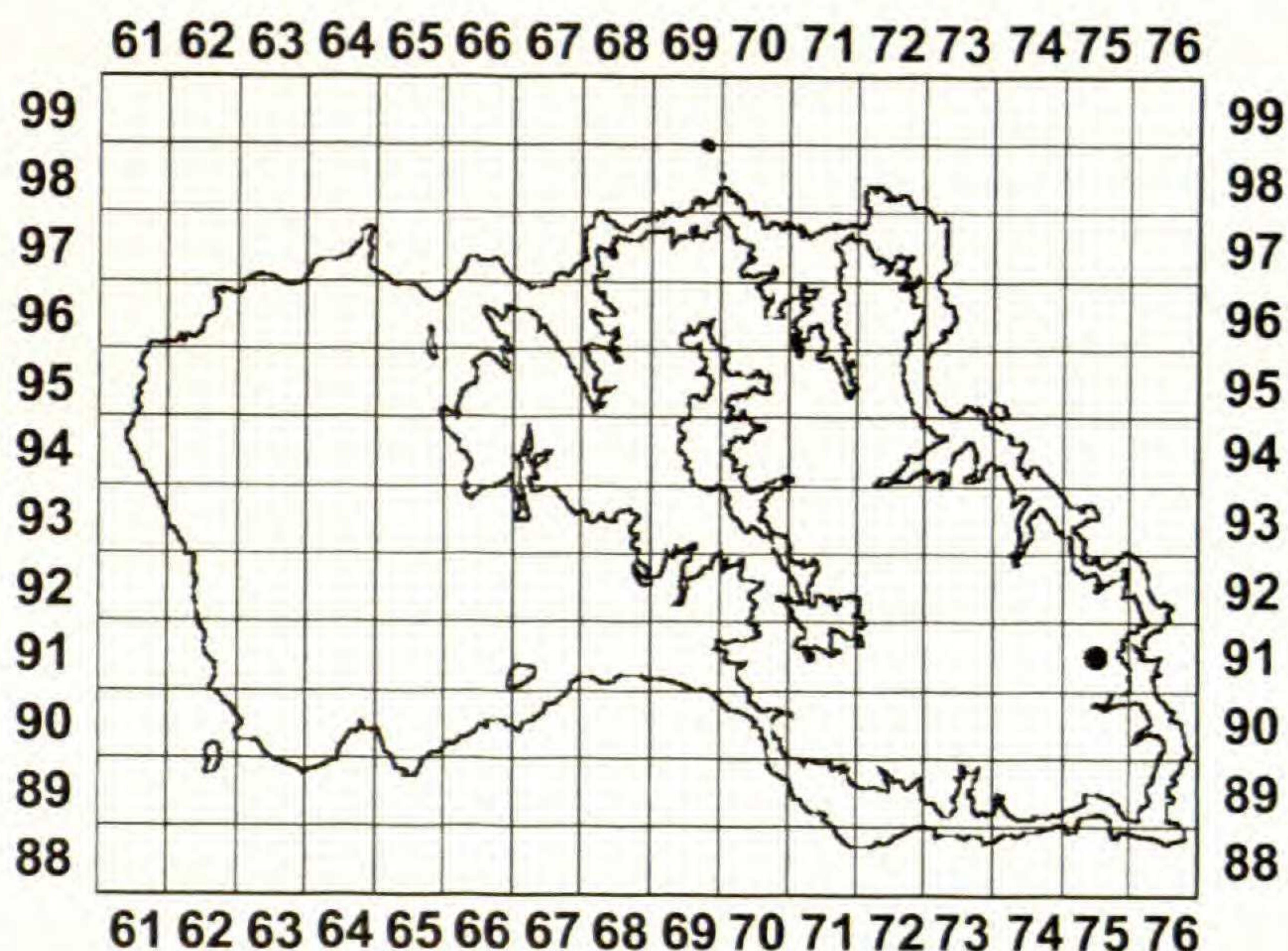


FIG. 7. Distribution of *Asplenium* \times *santamariae* on Santa Maria island in UTM 1 \times 1 km grid, WGS 84 (contour lines along 200 m isohypses).

Planta hybrida ex *A. azoricum* et *A. scolopendrium* exorta; rhizoma caespitosa; folia 10–15 cm longa, 2 cm lata; petiolus 3–5 cm longus, fuscus; rachis basi ferrugineo-fusca; pinnae cordatae-ovatae, 10 \times 9 mm, crispatae; superiores approximatae; sporae omnes abortivae.

Fronds 10–15 cm; stipe ca 5 cm, rachis ca 8 cm, black except uppermost 2 cm; pinnae up to 33, slightly crispate, confluent and crowded towards the tip of the frond; margin entire to shallowly dentate up to 10 \times 9 mm, ovate-elliptical to cordate, light green; sori ca 1 mm. Hybrid of *A. azoricum* and *A. scolopendrium* (the latter the maternal parent) with abortive spores. Given the ploidy levels of the two parents this taxon is assumed to be triploid. Fig. 5b.

In appearance *A. x santamariae* very closely resembles *A. x confluens* T. Moore, the extremely rare hybrid between *A. trichomanes* ssp. *quadrivalens* and *A. scolopendrium*. It differs from that hybrid in its less distinctly stalked pinnules with more crenulate-dentate margins and a somewhat thicker overall texture. As with *A. x diasii*, it is clear that the *A. trichomanes* group parent present in this hybrid is *A. azoricum*, it being the only simply pinnate species present. The confluent tip and broader, more decurrent pinnule attachments clearly indicate that the second parent of this hybrid would have fronds less divided than that of *A. azoricum*. The only Macaronesian species with less divided fronds are *A. scolopendrium* and *A. hemionitis* L. The latter is very rare on Santa Maria and has never been reported to hybridize with other species. It has a very distinctive, acutely lobed, palmate leaf, no sign of which is present in *A. x santamariae*. The participation of *A. scolopendrium*, in the origin of *A. x santamariae* is confirmed by examination of the hybrid's plastid genome.

Only one small individual of this hybrid was found in the archipelago. It grows in the south-eastern part of Santa Maria island (Fig. 7). The hybrid was

found in a large mixed population of the parents growing on a steep, west exposed slope with some *Pittosporum undulatum*. The slope and the *Pittosporum* stands are part of a large pasture that is, in some places, heavily grazed by cattle. Due to the animals and strong rainfall, erosion is a common phenomenon on the steep slopes. The resulting bare soil is soon colonized by a number of *Asplenium* species, including *A. adiantum-nigrum*, *A. azoricum*, *A. marinum*, *A. obovatum* ssp. *lanceolatum*, *A. onopteris*, and *A. scolopendrium*. Similar conditions, with periodic disturbance of soil banks, previously has been shown to promote hybrid formation between *Asplenium* species (Jee, 1994).

DISCUSSION AND CONCLUSIONS

The endemic *Asplenium azoricum* is widely distributed throughout the Azorean archipelago and is the most common representative of the *A. trichomanes* group on these islands. As it is able to survive even in dense plantations of invasive species, it cannot be considered endangered, although on those islands where it is very rare, e.g., Corvo, its few localities should be afforded protection. It is one of several fern species endemic to the Macaronesian region that almost certainly have evolved in the region but are not now sympatric with both their putative parents. It is hoped that ongoing phylogenetic studies will elucidate the relationship of *A. azoricum* and *A. trichomanes* ssp. *coriaceifolium* to the, as yet unknown, non-European parent they may share.

The merit of attempting to protect sterile fern hybrids is contentious, especially when resources are limited and many other endemic species are under threat, as in the Azores. Often the formation of the hybrids has been dependant on disturbance to sites through human activities. Arguably, if conditions and healthy populations of the parental taxa are maintained then it is likely that hybrids will continue to be sporadically produced. However, a strong case can be made for their conservation as unique biological entities with considerable evolutionary potential. Hybridization followed by polyploidy is the most rapid route to the generation of novel species and would seem to be the prevalent mode of speciation within the pteridophyta. These natural hybrids give us a rare opportunity to observe the process of allopolyploid speciation and the mechanisms and controlling factors behind it.

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